

METHOD FOR PRODUCING L-METHIONINE BY FERMENTATION

Technical Field

5 The present invention relates to a method for producing L-methionine by fermentation. L-methionine is an important amino acid as a medicament and the like.

Background Art

10

 Industrially produced methionine mainly consists of DL-methionine, which is produced through chemical synthesis. When L-methionine is required, it is provided through production of N-acetyl-DL-methionine by
15 acetylation of DL-methionine and subsequent enzymatic selective deacetylation of the N-acetylated L-methionine.

 On the other hand, as for the production of L-methionine by fermentation, methods utilizing an L-methionine analogue-resistant mutant strain have been
20 reported. However, their production amount is small, and factors affecting the L-methionine production have not been elucidated yet. Therefore, L-methionine is still one of the amino acids the most difficult to be produced by fermentation. For example, while methods
25 utilizing *Escherichia coli* (*E. coli*) K-12 strain have been reported in Japanese Patent Laid-open (Kokai) No. 56-35992 and literature (Chattapadhyay, M.K. et al., Med.

Sci. Res. 23, 775 (1995); Chattopadhyay, M.K. et al., *Biotechnol. Lett.* 17, 567-570 (1995)), any of these methods cannot provide L-methionine production amount sufficient for industrial use.

- 5 In *E. coli*, the biosynthetic pathway of L-methionine is partly shared with the biosynthetic pathway of L-threonine, and L-homoserine serves as a common intermediate. The first step of the peculiar pathway from L-homoserine to L-methionine is catalyzed
- 10 by homoserine transsuccinylase (HTS). This enzyme has been known to suffer concerted inhibition by the final product, L-methionine, and a metabolite of L-methionine, S-adenosylmethionine (Lee, L.-W. et al., *J. Biol. Chem.*, 241, 5479-5480 (1966)).
- 15 The nucleotide sequence of the *metA* gene encoding homoserine transsuccinylase of *E. coli*, has been reported by Duclos et al. (Duclos, B. et al., *Nucleic Acids Res.* 17, 2856 (1989)), and a method for obtaining a strain having a mutation for *metA* using resistance to
- 20 an analog of L-methionine, α -methyl-DL-methionine (MM) has also been known (Chattopadhyay, M.K. et al., *J. Gen. Microbiol.*, 137, 685-691 (1991)). It has been reported for *Salmonella typhimurium* that, the *metA* gene product, homoserine transsuccinylase, was an inhibition-
- 25 desensitized type as for the inhibition by L-methionine and ~~S-adenosylmethionine~~ ^{S-adenosylmethionine synthase} (SAM) in an MM resistant strain (Lawrence, D.A. et al., *J. Bacteriol.*, 109, 8-11 (1972)).

However, the nucleotide sequence of the mutant *metA* gene has not been reported. Furthermore, it has been reported that a mutant having a sole mutation in *metA* did not secrete L-methionine (Chattopadhyay, M.K. et al.,
5 *J. Gen. Microbiol.*, 137, 685-691 (1991)).

It has also been revealed that the expression of the genes including *metA* of the enzymes for the reaction by homoserine transsuccinylase and subsequent reactions in the peculiar biosynthetic pathway of L-methionine
10 suffers inhibition by a repressor which is a *metJ* gene product (Green, R.C. Biosynthesis of Methionine in "*Escherichia coli* and *Salmonella* Cellular and Molecular Biology/Second Edition", ed. Neidhardt, F.D., ASM Press, pp.542-560 (1996)). It has also been known that the
15 *metJ* gene is adjacent to the *metBL* operon in a reverse direction, which operon consists of the *metB* gene coding for the second enzyme of the peculiar biosynthetic pathway for L-methionine, cystathionine γ -synthase, and *metL* coding for aspartokinase-homoserine dehydrogenase
20 II (AK-HDII) (Duchange, N. et al., *J. Biol. Chem.*, 258, 14868-14871 (1983)).

It has been suggested that *metK* coding for S-adenosylmethionine, which catalyzes the metabolic reaction from L-methionine to S-adenosylmethionine,
25 should be an essential enzyme (Green, R.C. Biosynthesis of Methionine in "*Escherichia coli* and *Salmonella* Cellular and Molecular Biology/Second Edition", ed.

a' Neidhardt, F.D., ASM Press, pp.542-560 (1996)).

Furthermore, it has also been known that a ^{metK}~~metJ~~ mutant strain can be obtained based on resistance to a methionine analogue such as DL-norleucine and ethionine
5 (Chattopadhyay, M.K. et al., *J. Gen. Microbiol.*, 137, 685-691 (1991)), and can increase the expression of the enzymes of the peculiar biosynthetic pathway of L-methionine (Greene, R.C. et al., *J. Bacteriol.*, 115, 57-67).

10 As mentioned above, there have been reported enzymes involved in the L-methionine biosynthesis and genes therefor to some extent. However, only few findings that directly lead to the production of L-methionine by fermentation have been obtained, and hence
15 hardly applied to breeding of L-methionine-producing bacteria.

Summary of the Invention

20 The present invention has been accomplished in view of the aforementioned current technical status, and an object of the present invention is to elucidate factors affecting the L-methionine production, thereby breeding L-methionine-producing bacteria, and enabling
25 L-methionine production by fermentation.

In order to achieve the aforementioned object, the present inventors earnestly conducted studies, and as a

result, accomplished the present invention.

That is, the present invention provides:

(1) A microorganism which is deficient in repressor of L-methionine biosynthesis system and has L-methionine productivity;

(2) a microorganism having enhanced intracellular homoserine transsuccinylase activity and L-methionine productivity;

(3) a microorganism which is deficient in repressor of L-methionine biosynthesis system, and has enhanced intracellular homoserine transsuccinylase activity and L-methionine productivity;

(4) the microorganism according to any one of the above (1)-(3), which further exhibits reduced intracellular S-adenosylmethionine synthetase activity;

(5) the microorganism according to any one of the above (2)-(4), wherein the enhanced intracellular homoserine transsuccinylase activity is obtained by increasing copy number of a gene encoding homoserine transsuccinylase, or enhancing an expression regulatory sequence for the gene;

(6) the microorganism according to the above (1) or (4), which has homoserine transsuccinylase for which concerted inhibition by L-methionine and S-adenosylmethionine is desensitized;

(7) the microorganism according to any one of the above (1)-(6), which exhibits L-threonine

auxotrophy;

(8) the microorganism according to any one of the above (1)-(7), which exhibits enhanced intracellular cystathionine γ -synthase activity and enhanced
5 intracellular aspartokinase-homoserine dehydrogenase II activity;

(9) the microorganism according to any one of the above (1)-(8), which belongs to the genus *Escherichia*;

10 (10) a method for producing L-methionine which comprises culturing the microorganism according to any one of the above (1)-(9) in a medium to produce and accumulate L-methionine in the medium, and collecting the L-methionine from the medium; and

15 (11) A DNA which codes for homoserine transsuccinylase for which concerted inhibition by L-methionine and S-adenosylmethionine is desensitized, wherein the homoserine transsuccinylase has the amino acid sequence of SEQ ID NO: 26 including a mutation
20 corresponding to replacement of arginine by cysteine at the 27th position, mutation corresponding to replacement of isoleucine by serine at the 296th position, mutation corresponding to replacement of proline by leucine at the 298th position, mutation corresponding to
25 replacement of arginine by cysteine at the 27th position and replacement of isoleucine by serine at the 296th position, mutation corresponding to replacement of

isoleucine by serine at the 296th position and replacement of proline by leucine at the 298th position, mutation corresponding to replacement of proline by leucine at the 298th position and replacement of arginine by cysteine at the 27th position, or mutation corresponding to replacement of arginine by cysteine at the 27th position, replacement of isoleucine by serine at the 296th position and replacement of proline by leucine at the 298th position.

10 In this specification, S-adenosylmethionine will occasionally be abbreviated as "SAM", α -methyl-DL-methionine as "MM", and DL-norleucine as "NL". Further, S-adenosylmethionine synthetase will be occasionally be abbreviated as "SAM synthetase", and homoserine transsuccinylase as "HTS". The *metB* gene product, 15 cystathionine γ -synthase, of *E. coli* may also be called as "cystathionine synthase", and the *metL* gene product, "aspartokinase homoserine dehydrogenase II", may also be called as AK-HDII.

20 The term "L-methionine productivity" used for the present invention means an ability to accumulate L-methionine in a medium when a microorganism is cultured in the medium.

According to the present invention, there is 25 provided a microorganism having L-methionine production ability. The microorganism can be utilized as an L-methionine-producing bacterium or a material for

breeding of L-methionine-producing bacteria.

The mutant *metA* gene of the present invention can be utilized for the breeding of L-methionine-producing bacteria, because the concerted inhibition by L-methionine and SAM for the enzyme encoded by it is canceled.

Detailed Description of the Invention

10 Hereafter, the present invention will be explained in detail.

The microorganism of the present invention is a microorganism which is deficient in repressor of the L-methionine biosynthesis system and has L-methionine productivity, or a microorganism which has enhanced intracellular homoserine transsuccinylase activity and L-methionine productivity. The microorganism of the present invention is preferably a microorganism which is deficient in repressor of the L-methionine biosynthesis system, and has enhanced intracellular homoserine transsuccinylase activity. The microorganism of the present invention further preferably exhibits reduced intracellular SAM synthetase activity.

The aforementioned microorganism of the present invention is not particularly limited, so long as it has a pathway for producing L-methionine and SAM from L-homoserine via O-acylhomoserine which is produced from

L-homoserine by the acyl-transferring reaction, and its expression of the acyl transferase is controlled through suppression by a repressor. While such a microorganism may be an *Escherichia* bacterium, coryneform bacterium, and *Bacillus* bacterium, it is preferably an *Escherichia* bacterium, for example, *E. coli*.

If the microorganism of the present invention is a bacterium in which HTS possessed by the microorganism suffers concerted inhibition by SAM and L-methionine like *E. coli*, its L-methionine productivity may be improved by canceling the inhibition.

As the peculiar pathway for the methionine biosynthesis, there are one which uses cystathionine as an intermediate as in many microorganisms such as *E. coli*, and one which does not use cystathionine as in *Brevibacterium flavum* (Ozaki, H. et al., *J. Biochem.*, 91, 1163 (1982)). In the present invention, a microorganism that uses cystathionine is preferred. In such a microorganism, L-methionine productivity can be increased by enhancing the intracellular cystathionine synthase activity. In addition, even if it is a microorganism like *Brevibacterium flavum*, L-methionine productivity may be enhanced by deficiency of repressor in the L-methionine biosynthesis system and/or enhancement of HTS.

Furthermore, in the aforementioned microorganism, L-methionine productivity can further be increased by

enhancing at least one of the aspartokinase activity and the homoserine dehydrogenase activity, which are involved in the shared pathway of the L-methionine biosynthesis and the L-threonine biosynthesis.

5 When two or more of the aforementioned characteristics are imparted to a microorganism, the order for imparting them is not particularly limited, and they can be given in an arbitrary order. Moreover, when multiple genes are introduced into a microorganism,
10 those genes may be carried by the same vector, or may be separately carried by multiple different vectors. When multiple vectors are used, it is preferred to use vectors having different drug markers and different replication origins.

15 Methods for imparting each of the aforementioned characteristics to a microorganism will be explained below.

<1> Deficiency of repressor in L-methionine biosynthesis
20 system

A microorganism deficient in a repressor in the L-methionine biosynthesis system can be obtained by subjecting microorganisms to a mutagenic treatment, and selecting a strain no longer producing the repressor.
25 The mutagenic treatment can be performed with means usually used for obtaining microbial mutants, for example, UV irradiation or treatment with an agent used

for mutagenesis such as N-methyl-N'-nitrosoguanidine (NTG) and nitrous acid.

The repressor can also be made deficient by destroying a gene coding for the repressor on
5 chromosomal DNA of the microorganism. The gene can be destroyed by preparing a deleted type gene which has deletion of at least a part of a coding region or expression regulatory sequence, and causing homologous recombination of the deleted type gene and a gene on the
10 chromosome to substitute the deleted type gene for the gene on the chromosome (gene substitution).

Since the nucleotide sequence of the gene coding for the repressor in the L-methionine biosynthesis system of *E. coli* (*metJ*) has been known (Duchange, N. et
15 al., *J. Biol. Chem.*, 258, 14868-14871 (1983)), the repressor can be isolated from chromosomal DNA, for example, by PCR using primers produced based on the nucleotide sequence. A deleted type gene can be obtained by excising a certain region from the gene
20 fragment obtained as described above, and deleting at least a part of the coding region or expression regulatory region.

The gene substitution can be performed, for example, as follows. A deleted type gene is introduced
25 into a vector having a temperature sensitive replication origin to prepare a recombinant vector, and a microorganism is transformed with the recombinant vector

so that the deleted type gene should be inserted into a gene on chromosomal DNA by homologous recombination of the deleted type gene and the gene on the chromosomal DNA. Then, the transformant strain is cultured at a temperature at which the vector cannot replicate to drop out the vector from cytoplasm. Furthermore, the gene is replaced when one copy of the gene on the chromosome is dropped out with the vector. The occurrence of the desired gene substitution can be confirmed by Southern hybridization analysis of the chromosomal DNA of a strain to be tested for the gene substitution.

As a vector for *E. coli* that has a temperature sensitivity replication origin, for example, the plasmid pMAN997 disclosed in Japanese Patent Application No. 9-194603 and the like can be mentioned. As a vector for coryneform bacteria that has a temperature sensitivity replication origin, for example, the plasmid pHSC4 disclosed in Japanese Patent Laid-open No. 5-7491 and the like can be mentioned. However, the vector is not limited to these, and other vectors can also be used.

As mentioned above, it has known for *E. coli* that the *metJ* gene is adjacent to the *metBL* operon, which consists of *metB* gene and *metL* gene, in the reverse direction (Duchange, N. et al., *J. Biol. Chem.*, 258, 14868-14871 (1983)). Therefore, if a suitable promoter sequence is ligated to a deleted type *metJ* gene and gene substitution is performed as described above, the

destruction of the *metJ* gene and improvement of expression utilizing substitution of promoter in the *metBL* operon can simultaneously be obtained by one homologous recombination. Improved expression of the
5 *metBL* operon enhances the intracellular cystathionine synthase activity and AK-HDII activity.

Specifically, the following three components, a fragment of about 1 kb containing the *metB* gene, which is obtained by, for example, PCR (polymerase chain
10 reaction; White, T. J. et al.; *Trends Genet.*, 5, 185 (1989)) utilizing chromosomal DNA of *E. coli* W3110 strain as a template, and oligonucleotides having the nucleotide sequences of SEQ ID NO: 5 and SEQ ID NO: 6 as primers; a fragment of about 1 kb containing the
15 downstream region of the *metJ* gene obtained by PCR utilizing oligonucleotides having the nucleotide sequences of SEQ ID NO: 7 and SEQ ID NO: 8 as primers; and a sequence having a promoter sequence of the threonine operon, which is obtained by annealing of the
20 oligonucleotides represented as SEQ ID NO: 9 and SEQ ID NO: 10, can be inserted into a suitable vector, and ligating to it to obtain a recombinant vector which contains a DNA fragment having deletion of the structural gene of *metJ* and substitution of threonine
25 promoter for the promoter of the *metBL* operon.

To introduce the recombinant DNA prepared as described above to bacterium, any known transformation

methods can be employed. For instance, employable are a method of treating recipient cells with calcium chloride so as to increase the permeability of DNA, which has been reported for *Escherichia coli* K-12 [see Mandel, M. and Higa, A., *J. Mol. Biol.*, 53, 159 (1970)]; and a method of preparing competent cells from cells which are at the growth phase followed by introducing the DNA thereinto, which has been reported for *Bacillus subtilis* [see Duncan, C.H., Wilson, G.A. and Young, F.E., *Gene*, 1, 153 (1977)]. Alternatively, it is also possible to apply a method in which DNA recipient cells are allowed to be in a state of protoplasts or spheroplasts capable of incorporating recombinant DNA with ease to introduce recombinant DNA into the DNA recipient cells, as known for *Bacillus subtilis*, actinomycetes, and yeasts (Chang, S. and Choen, S. N., *Molec. Gen. Genet.*, 168, 111 (1979); Bibb, M. J., Ward, J. M. and Hopwood, O. A., *Nature*, 274, 398 (1978); Hinnen, A., Hicks, J. B. and Fink, G. R., *Proc. Natl. Acad. Sci. USA*, 75, 1929 (1978)). Transformation of coryneform bacteria may be performed by the electric pulse method (refer to Japanese Patent Publication Laid-Open No. 2-207791).

The vector to be used for cloning the genes such as *metA*, *metK* and *thrBC* as described below includes, for example, pUC19, pUC18, pBR322, pHSG299, pHSG399, pHSG398, RSF1010 and the like. Besides, it is possible to use phage DNA vectors. When microorganisms other than *E.*

coli are used, it is preferable to use a shuttle vector autonomously replicable in those microorganisms and *E. coli*. As examples of plasmid autonomously replicable in coryneform bacteria, for example, the followings can be
5 mentioned.

pAM330 (see Japanese Patent Laid-open No. 58-67699)

pHM1519 (see Japanese Patent Laid-open No. 58-77895)

10 pAJ655 (see Japanese Patent Laid-open No. 58-192900)

pAJ611 (see the same)

pAJ1844 (see the same)

15 pCG1 (see Japanese Patent Laid-open No. 57-134500)

pCG2 (see Japanese Patent Laid-open No. 58-35197)

pCG4 (see Japanese Patent Laid-open No. 57-183799)

20 pCG11 (see the same)

pHK4 (see Japanese Patent Laid-open No. 5-7491)

In order to prepare recombinant DNA by ligating the gene fragment and a vector, the vector is digested by restriction enzyme(s) corresponding to the termini of
25 the gene fragment. Ligation is generally performed by using a ligase such as T4 DNA ligase.

The methods to perform, for example, preparation

of the genomic DNA library, hybridization, PCR, preparation of plasmid DNA, digestion and ligation of DNA, and design of oligonucleotide used for primers are described by Sambrook, J., Fritsche, E. F., Maniatis, T.
5 in Molecular Cloning, Cold Spring Harbor Laboratory Press (1989).

<2> Enhancement of HTS activity and introduction of mutant HTS

10 The HTS activity in a microbial cell can be attained by preparing a recombinant DNA through ligation of a gene fragment encoding HTS with a vector which functions in the microorganism, preferably a multi-copy type vector, and transforming the microorganism through
15 introduction of the plasmid into in the microbial cell. As a result of increase of the copy number of the gene encoding HTS in the transformant strain, the HTS activity is enhanced. In *E. coli*, HTS is encoded by the *metA* gene. When an *Escherichia* bacterium is used as the
20 microorganism, the HTS gene to be introduced is preferably a gene derived from an *Escherichia* bacterium. However, genes derived from other microorganisms having homoserine transacetylase, such as coryneform bacteria, can also be used.

25 Enhancement of HTS activity can also be achieved by introducing multiple copies of the HTS gene into the chromosomal DNA of the above-described host strains. In

order to introduce multiple copies of the HTS gene in the chromosomal DNA of bacterium belonging to the genus *Corynebacterium*, the homologous recombination is carried out using a sequence whose multiple copies exist in the chromosomal DNA as targets. As sequences whose multiple copies exist in the chromosomal DNA, repetitive DNA, inverted repeats exist at the end of a transposable element can be used. Also, as disclosed in Japanese Patent Laid-open No. 2-109985, it is possible to incorporate the HTS gene into transposon, and allow it to be transferred to introduce multiple copies of the HTS gene into the chromosomal DNA. By either method, the number of copies of the HTS gene within cells of the transformant strain increases, and as a result, HTS activity is enhanced.

The enhancement of HTS activity can also be obtained by, besides being based on the aforementioned gene enhancement, enhancing an expression regulatory sequence for the HTS gene. Specifically, it can be attained by replacing an expression regulatory sequence of HTS gene on chromosome DNA or plasmid, such as a promoter, with a stronger one (see Japanese Patent Laid-open No. 1-215280). For example, lac promoter, trc promoter, tac promoter, PR promoter and PL promoter of lambda phage and the like are known as strong promoters. Substitution of these promoters enhances expression of the HTS gene, and hence the HTS activity is enhanced.

Since the nucleotide sequence of the HTS gene (metA) of *E. coli* has been known (Blattner, F.R. et al., *Science*, 277, 1453-1462 (1997)), it can be isolated from chromosomal DNA by PCR using primers produced based on
5 the nucleotide sequence. As such primers, the oligonucleotides having the nucleotide sequences represented as SEQ ID NO: 21 and SEQ ID NO: 22 are specifically mentioned.

It is expected that, by enhancing the HTS activity
10 in a microbial cell as described above, L-methionine biosynthesis can be enhanced, and thus the L-methionine production amount can be increased.

Further, because HTS suffers concerted inhibition by L-methionine and SAM, the L-methionine biosynthesis
15 system can also be enhanced by obtaining a microorganism containing HTS for which concerted inhibition has been canceled. Such a microorganism containing HTS for which concerted inhibition has been canceled can be obtained by subjecting microorganisms to a mutagenic treatment,
20 and selecting a strain containing HTS for which concerted inhibition has been canceled. The mutagenic treatment can be performed with means usually used for obtaining microbial mutants, for example, UV irradiation or treatment with an agent used for mutagenesis such as
25 N-methyl-N'-nitrosoguanidine (NTG) and nitrous acid. The expression "HTS for which concerted inhibition has been canceled" herein used means HTS exhibiting a ratio

of its enzymatic activity in the presence of L-methionine, SAM or L-methionine and SAM to its enzymatic activity in the absence of L-methionine and SAM (remaining ratio) higher than that of a wild-type HTS.

5 Specifically, an HTS exhibiting a remaining ratio in the presence of 1 mM L-methionine of 40% or more, preferably 80% or more, a remaining ratio in the presence of 1 mM SAM of 10% or more, preferably 50% or more, or a remaining ratio in the presence of 0.1 mM each of L-

10 methionine and SAM of 15% or more, preferably 60% or more is an HTS for which concerted inhibition by L-methionine and SAM has been canceled.

A mutant strain having such a mutant HTS as mentioned above can be obtained by culturing a parent

15 strain in the presence of α -methyl-DL-methionine (MM), e.g., in a medium containing 1 g/l of MM, and selecting a strain growing on the medium. The selection with MM may be repeated two or more times.

A mutant strain having a mutant HTS can also be

20 obtained by cloning the mutant HTS gene (mutant *metA*) from a HTS mutant obtained as described above, and transforming a microorganism with the mutant gene. Isolation of a mutant HTS gene and introduction of the gene into a microorganism can be performed as the

25 aforementioned wild-type HTS gene. As HTS having a mutant *metA* gene, HTS having the amino acid sequence of SEQ ID NO: 26 including a mutation corresponding to

replacement of arginine by cysteine at the 27th position, mutation corresponding to replacement of isoleucine by serine at the 296th position, or mutation corresponding to replacement of proline by leucine at the 298th position can specifically be mentioned. HTS including two or more of these mutations is also a preferred mutant HTS.

<3> Attenuation of SAM synthetase activity

Furthermore, the L-methionine productivity of microorganism can be increased by attenuating intracellular SAM synthetase activity. The L-methionine productivity of a microorganism can also be increased by making the microorganism SAM synthetase activity deficient, but in such a case, the medium for culturing the microorganism must contain SAM. Therefore, it is preferable to attenuate SAM synthetase activity. The expression "attenuating SAM synthetase activity" herein used means that a specific activity of SAM synthetase per unit of protein in microbial cells is made lower than that of a strain having a wild-type SAM synthetase. Specifically, the degree of attenuation, i.e., the reduced activity may be 80% to 50%, preferably 50% to 30%, more preferably 30% to 10% of the SAM synthetase activity of a wild-type strain. In *E. coli*, it has been suggested that, if the specific activity of SAM synthetase falls below 10%, cell division would be

inhibited (Newman, E. B. et al., *J. Bacteriol.*, 180, 3614-3619 (1998)).

The microorganism whose SAM synthetase activity is reduced may be one producing SAM synthetase exhibiting a
5 reduced specific activity per enzymatic protein (reduced type SAM synthetase), or one in which expression efficiency of the enzyme is reduced because of reduced transcription efficiency or reduced translation efficiency of SAM synthetase gene.

10 A mutant strain whose SAM synthetase activity is reduced may be obtained by culturing a parent strain in the presence of DL-norleucine (NL), e.g., in a medium containing 0.1 g/l of NL, and selecting a grown strain. The selection with NL may be repeated two or more times.
15 It is also possible to use ethionine or γ -glutamylmethyl ester instead of DL-norleucine.

A mutant strain having an reduced type SAM synthetase can also be obtained by cloning a gene for the reduced type SAM synthetase from a SAM synthetase-
20 reduced strain obtained as described above, and substituting the mutant gene for a wild-type SAM synthetase gene on a chromosome of microorganism. The gene substitution of SAM synthetase gene can be performed in the same manner as the aforementioned *metJ*
25 gene. Since the nucleotide sequence of the SAM synthetase gene (*metK*) of *E. coli* has been known (Blattner, F.R. et al., *Science*, 277, 1453-1462 (1997)),

it can be isolated from chromosomal DNA by PCR using primers produced based on the nucleotide sequence. As such primers, the oligonucleotides having the nucleotide sequences represented as SEQ ID NO: 11 and SEQ ID NO: 12 are specifically mentioned. The occurrence of the desired mutation in the obtained *metK* gene can be confirmed by determining the nucleotide sequence of the gene, and comparing it with a known nucleotide sequence of wild-type *metK* gene.

As specific examples of the gene coding for an reduced type SAM synthetase, those coding for SAM synthetases having the amino acid sequence of SEQ ID NO: 18 including a mutation corresponding to replacement of isoleucine by leucine at the 303rd position, mutation corresponding to replacement of valine by glutamic acid at the 185th position, or mutation corresponding to replacement of arginine at the 378th position and subsequent amino acid residues by a sequence of alanine-methionine-leucine-proline-valine (SEQ ID NO: 29) can be mentioned.

<4> L-Threonine auxotrophy

L-methionine productivity can be improved by imparting L-threonine auxotrophy to a microorganism. Specific examples of a microorganism exhibiting L-threonine auxotrophy include those having deficiency of any one of enzymes involved in the peculiar pathway of

L-threonine biosynthesis from L-homoserine to L-threonine. In *E. coli*, the genes of the enzymes involved in the biosynthesis of L-threonine exist as the threonine operon (*thrABC*), and L-threonine auxotrophic
5 strain, which has lost the ability to synthesize L-homoserine and subsequent products, can be obtained by deleting the *thrBC* segment. The *thrA* gene codes for one of the isozymes of aspartokinase, which is an enzyme of the shared pathway of the L-methionine and L-threonine
10 biosyntheses, and hence it is preferably not to be deleted.

In order to delete *thrBC*, the *thrBC* segment in the threonine operon on chromosomal DNA can be destroyed. *thrBC* can be destroyed by replacing the *thrBC* segment on
15 a microbial chromosome with *thrBC* a part of which is deleted. The gene substitution of *thrBC* may be performed in the same manner as in the gene substitution of the aforementioned *metJ* gene. A *thrBC* segment containing deletion can be obtained by amplifying a
20 fragment of about 1 kb containing the upstream region of the *thrB* gene by PCR using *E. coli* chromosomal DNA as a template and primers having the nucleotide sequences of SEQ ID NOS: 1 and 2, similarly amplifying a fragment of about 1 kb containing the downstream region of the *thrC*
25 gene by PCR using primers having the nucleotide sequences of SEQ ID NOS: 3 and 4, and ligating these amplified products.

<5> Production of L-methionine

L-methionine can be produced by culturing a microorganism having L-methionine productivity prepared as described above in a medium so that L-methionine should be produced and accumulated in the medium, and collecting L-methionine from the medium.

The medium to be used may be selected from well-known media conventionally used depending on the kind of microorganism to be used. That is, it may be a usual medium that contains a carbon source, nitrogen source, inorganic ions, and other organic ingredients as required. Any special medium is not needed for the practice of the present invention.

As the carbon source, it is possible to use sugars such as glucose, lactose, galactose, fructose or starch hydrolysate; alcohols such as glycerol or sorbitol; or organic acids such as fumaric acid, citric acid or succinic acid.

As the nitrogen source, it is possible to use inorganic ammonium salts such as ammonium sulfate, ammonium chloride or ammonium phosphate; organic nitrogen such as soybean hydrolysate; ammonia gas; or aqueous ammonia.

It is desirable to allow required substances such as vitamin B1, L-threonine and L-tyrosine or yeast extract to be contained in appropriate amounts as

organic trace nutrients. Other than the above, potassium phosphate, magnesium sulfate, iron ion, manganese ion and the like are added in small amounts, if necessary.

5 Cultivation is preferably carried out under an aerobic condition for 16-120 hours. The cultivation temperature is preferably controlled at 25°C to 45°C, and pH is preferably controlled at 5-8 during cultivation. Inorganic or organic, acidic or alkaline
10 substances as well as ammonia gas or the like can be used for pH adjustment.

Any special techniques are not required for collecting L-methionine from the medium after the cultivation in the present invention. That is, the
15 present invention can be practiced by a combination of well-known techniques such as techniques utilizing ion exchange resin and precipitation.

Best Mode for Carrying out the Invention

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The present invention will further be explained more specifically with reference to the following examples.

25 Example 1: Acquisition of L-threonine auxotrophic strain and metJ deficient strain from Escherichia coli W3110 strain

<1> Preparation of plasmid for recombination containing *thrBC* structural gene having deletion

Chromosomal DNA was prepared from W3110 strain,
5 which was a derivative of the wild-type K-12 strain of *E. coli*, by using a genomic DNA purification kit (Advanced Genetic Technology) according to the instruction of the kit. Oligonucleotides having the nucleotide sequences of SEQ ID NO: 1 and SEQ ID NO: 2 in Sequence Listing
10 were synthesized. PCR was performed according to the method of Erlich et al. (PCR Technology-Principles and Applications for DNA Amplification, ed. Erlich, H.A., Stockton Press) by using the above oligonucleotides as primers and the aforementioned chromosomal DNA as the
15 template to amplify a fragment of about 1 kb containing the upstream region of the *thrB* gene. This amplification fragment was introduced with recognition sequences for *EcoRI* and *SalI*, which were derived from the primers. The obtained amplified fragment was
20 digested with restriction enzymes corresponding to the introduced recognition sites.

Similarly, PCR was performed by using oligonucleotides having the nucleotide sequences of SEQ ID NO: 3 and SEQ ID NO: 4 as primers to amplify a
25 fragment of about 1 kb containing the downstream region of the *thrC* gene. This amplification fragment was introduced with recognition sequences for *SalI* and

*Hind*III, which were derived from the primers. The obtained amplified fragment was digested with restriction enzymes corresponding to the introduced recognition sites. The aforementioned two amplified
5 fragments, and pHSG398 (TAKARA SHUZO) digested with *Eco*RI and *Hind*III were ligated by using a ligation kit (TAKARA SHUZO), and *E. coli* JM109 competent cells (TAKARA SHUZO) were transformed with the ligation product. Plasmids were prepared from the transformants
10 based on the alkaline method (Boirnboim, H.C. et al., *Nucleic Acids Res.*, 7, 1513-1523 (1979)) by using a Plasmid Extractor PI-50 (Kurabo Industries, Ltd.). From the obtained plasmids, a plasmid in which two fragments were inserted in the *Eco*RI and *Hind*III recognition sites
15 through *Sal*I recognition sites was selected based on the lengths of inserted fragments. This plasmid contained the upstream and the downstream regions of the structural gene of *thrBC*, and contained a gene fragment in which substantially full length of the structural
20 gene of *thrBC* was deleted.

<2> Production of *thrBC* structural gene deletion strain by genetic recombination

The aforementioned plasmid and the plasmid pMAN997
25 having a temperature sensitive replication origin, which was disclosed in Japanese Patent Application No. 9-194603, were digested with *Eco*RI and *Hind*III, and

ligated each other. The *E. coli* JM109 strain was transformed with the obtained recombinant plasmid. Plasmids were extracted from the transformants, and one having a structure where a *thrBC*-deleted gene fragment was inserted in pMAN997 was selected, and designated as pMAN Δ BC. The W3110 strain was transformed with this plasmid to perform genetic recombination in a conventional manner. That is, selection of recombinant strains was carried out based on the L-threonine auxotrophy in M9 medium (Sambrook, J. et al., "Molecular Cloning: A Laboratory Manual/Second Edition", Cold Spring Harbor Laboratory Press, A.3 (1989)), and the obtained L-threonine auxotrophic strain was designated as W Δ BC strain.

15

<3> Production of *metJ* deficient strains from W3110 strain and W Δ BC strain

Then, PCR was performed by using the W3110 strain chromosomal DNA as the template and oligonucleotides having the nucleotide sequences of SEQ ID NO: 5 and SEQ ID NO: 6 as primers to amplify a fragment of about 1 kb containing the *metB* gene. This amplification fragment was introduced with recognition sequences for EcoRI and SphI. The obtained amplified fragment was digested with restriction enzymes corresponding to the introduced recognition sites.

25

Similarly, PCR was performed by using

oligonucleotides having the nucleotide sequences of SEQ ID NO: 7 and SEQ ID NO: 8 as primers to amplify a fragment of about 1 kb containing the downstream region of the *metJ* gene. This amplification fragment was
5 introduced with recognition sequences for *SalI* and *HindIII*. The obtained amplified fragment was digested with restriction enzymes corresponding to the introduced recognition sites.

Then, a sequence represented as SEQ ID NO: 9,
10 which contained *SphI* and *HindIII* recognition sites at the both ends and the promoter sequence of the threonine operon, and its complementary strand represented as SEQ ID NO: 10 were synthesized, annealed, and digested with restriction enzymes *SphI* and *HindIII*. The threonine
15 promoter fragment obtained as described above, pHSG298 (TAKARA SHUZO) digested with *EcoRI*, and the aforementioned two PCR amplification fragments were mixed, and ligated. The JM109 strain was transformed with this ligation solution, and plasmids were extracted
20 from the transformants. A plasmid comprising ligated four of the components was selected from the obtained plasmids. This plasmid had a structure where the *metJ* structural gene was deleted, and the promoter of *metBL* operon was replaced with the threonine promoter.

25 The plasmid obtained above and the plasmid pMAN997 having a temperature sensitive replication origin, which is disclosed in Japanese Patent Application No. 9-194603,

were digested with *EcoRI*, and ligated each other. A plasmid having a structure where a *metJ*-deleted fragment was inserted into pMAN997 was selected, and designated as pMANΔ*J*. The W3110 strain and the WΔBC strain were transformed with this plasmid to perform genetic recombination in a conventional manner. Selection of recombinant strains was performed based on the lengths of amplified products from PCR utilizing DNA prepared from the cells as the template, and the oligonucleotides represented in SEQ ID NO: 6 and SEQ ID NO: 8 as primers. The *metJ*-deleted strains obtained from the W3110 strain and the WΔBC strain were designated as WΔ*J* strain and WΔBCΔ*J* strain, respectively.

In order to confirm the effect of the *metJ* deletion by the recombination, a crude enzyme extract was prepared from the cells, and the activities of HTS and cystathionine synthase were measured. The W3110 strain and the WΔ*J* strain were each inoculated to 2 ml of LB medium, and cultured at 37°C overnight. 1 ml of the medium was centrifuged at 5,000 rpm for 10 minutes, and the cells were washed twice with 0.9% saline. The obtained cell were suspended in 1 ml of 0.9% saline, 0.5 ml of which was inoculated to 50 ml of Davis-Mingioli minimal medium (Davis B.D., and Mingioli, E.S., *J. Bacteriol.*, 60, 17-28 (1950)) containing 5 mM L-methionine. The cells were cultured at 37°C for 24 hours, then the medium was centrifuged at 8,000 rpm for

10 minutes, and the cells were washed twice with 0.9% saline. The cells were suspended in 3 ml of 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM dithiothreitol. This suspension was subjected to a cell
5 disruption treatment at 4°C with a power of 150 W for 5 minutes by using an ultrasonicator (Kubota Co.). The sonicated suspension was centrifuged at 15,000 rpm for 30 minutes, and the supernatant was desalted in a Sephadex G-50 column (Pharmacia) to obtain a crude
10 enzyme extract. The HTS activity and the cystathionine synthase activity in the crude enzyme extract were measured.

As for the HTS activity, 5 μ l of the crude enzyme extract was added to a reaction mixture comprising 0.1 M
15 potassium phosphate (pH 7.5), 1 mM succinyl-coenzyme A (Sigma), 0.2 nM DL-[¹⁴C]homoserine (Muromachi Chemical Industry), and 0.2 mM L-homoserine to obtain a volume of 50 μ l, and allowed to react at 30°C for 10 minutes. 1 μ l of the reaction mixture was spotted on a cellulose
20 plate (Merck), and developed with a mixed solvent containing acetone, butanol, water, and diethylamine at a ratio of 10:10:5:2. After the plate was air-dried, autoradiography was performed by using an image analyzer (Fuji Photo Film).

25 The cystathionine synthase has been known to produce α -ketobutyric acid, ammonia, and succinic acid from O-succinylhomoserine in the absence of L-cysteine,

and this can be utilized for simple detection thereof (Holbrook, E.L. et al., *Biochemistry*, 29, 435-442 (1990)). 100 μ l of the crude enzyme extract was added to a reaction mixture comprising 0.2 M Tris-HCl (pH 8), 5 mM O-succinylhomoserine (Sigma), and 0.25 mM pyridoxal phosphate (Sigma) to obtain a volume of 1 ml, allowed to react at 37°C for 20 minutes, and cooled with ice. The O-succinylhomoserine in this reaction mixture was quantitated by reverse phase HPLC (GL Sciences), and the reduced amount of O-succinylhomoserines was calculated by using a reaction mixture not added the crude enzyme extract. The reaction was also performed with no addition of pyridoxal phosphate, and pyridoxal phosphate-dependent reduction of O-succinylhomoserine was defined to be the cystathionine synthase activity.

The specific activities for the HTS activity and the cystathionine synthase activity measured as described above were shown in Table 1. While the HTS activity was hardly detected in the W3110 strain due to the effect of L-methionine addition, marked activity was observed in the WΔJ strain. As also for the cystathionine synthase activity, remarkable increase was observed in the WΔJ strain compared with the W3110 strain. From these results, the effects of the *metJ* deletion and the promoter substitution in the *metBL* operon by recombination were confirmed.

Table 1: HTS activity and cystathionine synthase
activity in *metJ* deficient strain

Strain	HTS activity (mmol/min/mg protein)	Cystathionine synthase activity (mmol/min/mg protein)
W3110	0.3	140
WΔJ	126	1300

Example 2: Acquisition of *metK* mutant from W3110 strain

5

The W3110 strain was cultured in LB medium (Sambrook, J. et al., "Molecular Cloning: A Laboratory Manual/Second Edition", Cold Spring Harbor Laboratory Press, A.1 (1989)) at 37°C overnight. 1 ml of the
10 cultured medium was centrifuged at 5,000 rpm for 10 minutes, and the cells were washed twice with 0.9% saline. The obtained cells were suspended in 100 µl of 0.9% saline, 10 µl of which was inoculated to 5 ml of Davis-Mingioli minimal medium containing 0.1 g/l of DL-
15 norleucine (NL), and cultured at 37°C for 5 days.

Some of the grown colonies were subjected to colony separation on LB agar medium, and their growth was confirmed again in Davis-Mingioli minimal medium containing 0.1 g/l of NL to select 12 NL-resistant
20 strains. Chromosomal DNA was prepared from these resistant strains. PCR was performed by using this chromosomal DNA as a template and two sorts of primers having the sequences of SEQ ID NOS: 11 and 12 to amplify the *metK* gene. The nucleotide sequence of this

amplification fragment was determined by using
amplification primers of which sequences are shown as
SEQ ID NO: 11 and 12, and primers of which sequences are
shown as SEQ ID NOS: 13, 14, 15, and 16. The nucleotide
5 sequence determination was performed by using a Dye
Terminator Cycle Sequencing Kit (Perkin-Elmer) on a DNA
sequencer Model 373S (Perkin-Elmer) in accordance with
the instructions attached to them. The nucleotide
sequence of the wild strain W3110 determined as a
10 control completely coincided with the sequence of *metK*
reported by Blattner et al. (Blattner, F.R. et al.,
Science, 277, 1453-1462 (1997)). This sequence is
represented as SEQ ID NO: 17. Further, the amino acid
sequence of SAM synthetase which may be encoded by the
15 sequence of SEQ ID NO: 17 is shown in SEQ ID NO: 18.

Among the NL resistant strains, a mutation in the
structural gene of *metK* was found in 3 strains out of
the 12 strains, which were designated as WNL2, WNL24,
and WNL32. As for the *metK* nucleotide sequences of
20 these mutant strains, in the wild-type nucleotide
sequence shown as SEQ ID NO: 17, the WNL2 strain had
replacement of adenine by cytosine at the 907th position,
the WNL24 strain had replacement of thymine by adenine
at the 554th position, and the WNL32 strain had deletion
25 of cytosine at the 1132nd position. As a result, it was
found that, in the amino acid sequence of SAM synthetase
represented as SEQ ID NO: 18, the SAM synthetase of the

WNL2 strain had replacement of isoleucine by leucine at the 303rd position, that of the WNL24 strain had replacement of valine by glutamic acid at the 185th position, and that of the WNL32 strain had replacement of arginine at the 378th position and subsequent amino acid residues by alanine-methionine-leucine-proline-valine due to deletion of one nucleotide. It was estimated that the SAM synthetase activity was reduced in these strains.

10

Example 3: Production of L-methionine by introduction of *metK* mutation and amplification of wild-type *metA* gene

(1) Introduction of *metK* mutation into WABCAJ strain

15 PCR was performed by using each chromosomal DNA of the *metK* gene mutant strains, WNL2 strain, WNL24 strain, and WNL32 strain, as a template, and oligonucleotides of SEQ ID NO: 19 and SEQ ID NO: 20 as primers to amplify a fragment of about 2.5 kb containing the *metK* gene. This
20 amplification fragment was introduced with recognition sequences for *Hind*III at the both ends. The obtained amplified fragment was digested with *Hind*III. pSTV28 (TAKARA SHUZO) digested with *Hind*III and the PCR amplification fragment were mixed and ligated, and the
25 JM109 strain was transformed with the ligation product. Plasmids were extracted from the transformants. From the obtained plasmids, plasmids inserted with the PCR

amplification fragment were selected. As for these plasmids, mutations in the *metK* structural gene were confirmed by determining their nucleotide sequences.

*Hind*III digestion fragments of these plasmids were each cloned into pMAN997 digested with *Hind*III, and the resulting plasmids were designated as pMANK-2, pMANK-24, and pMANK-32, respectively. The W Δ BC Δ J strain was transformed with these plasmids to obtain genetic recombination in a conventional manner. Chromosomal DNA was extracted from the recombinant strains, and used as a template together with oligonucleotides having the nucleotide sequences of SEQ ID NO: 11 and SEQ ID NO: 12 as primers to perform PCR, and the amplification products were examined for nucleotide sequence to select those having mutations. The *metK* mutant strains obtained from the W Δ BC Δ J strain were designated as W Δ BC Δ JK-2 strain, W Δ BC Δ JK-24 strain, and W Δ BC Δ JK-32 strain, respectively.

(2) Amplification of *metaA* gene

PCR was performed by using W3110 strain chromosomal DNA as a template, and oligonucleotides having the nucleotide sequences of SEQ ID NO: 21 and SEQ ID NO: 22 as primers to amplify a fragment of about 1 kb containing the *metaA* gene. This amplification fragment was introduced with recognition sequences for *Sph*I and *Sal*I at the both ends. The obtained amplified fragment

was digested with restriction enzymes corresponding to the introduced recognition sites. The digested product was cloned into pHSG398 digested with *Sph*I and *Sal*I. The nucleotide sequence of the inserted fragment was
5 determined by using amplification primers represented as SEQ ID NOS: 21 and 22, and primers having the sequences of SEQ ID NOS: 23 and 24. The determined nucleotide sequence of *metaA* of the wild strain W3110 completely coincided with the sequence of *metaA* reported by Blattner
10 et al. (Blattner, F.R. et al., *Science*, 277, 1453-1462 (1997)). This sequence is represented as SEQ ID NO: 25. Further, the amino acid sequence of HTS which may be encoded by the sequence of SEQ ID NO: 25 is shown as SEQ ID NO: 26.

15 A *Sph*I and *Sal*I digestion product of this plasmid, *Hind*III and *Sph*I digestion product of the threonine promoter of Example 1, and pMW118 (Nippon Gene) digested with *Hind*III and *Sal*I were mixed and ligated. The JM109 strain was transformed with this reaction mixture, and
20 plasmids were extracted from the transformants. From the obtained plasmids, a plasmid in which the three components were ligated was selected. This plasmid had a structure where the *metaA* gene was positioned at the downstream from the threonine promoter, by which the
25 *metaA* was expressed. This plasmid was designated as pMWPthmetaA-W. The W3110 strain, WABC strain, WABCΔJ strain, WABCΔJK-2 strain, WABCΔJK-24 strain, and

WABCΔJK-32 strain were transformed with this plasmid to obtain transformants.

Each transformant was cultured at 37°C overnight on an LB plate containing 50 mg/l of ampicillin. The
5 cells were inoculated to 20 ml of medium at pH 7 containing 40 g/l of glucose, 1 g/l of magnesium sulfate, 16 g/l of ammonium sulfate, 1 g/l of potassium dihydrogenphosphate, 2 g/l of yeast extract (Bacto Yeast-Extract, Difco), 0.01 g/l of manganese sulfate,
10 0.01 g/l of iron sulfate, 30 g/l of calcium carbonate, 50 mg/l of ampicillin, and 0.5 g/l of L-threonine, and cultured at 37°C for 48 hours.

The cells were separated from the culture, and the amount of L-methionine was measured by an amino acid
15 analyzer (Hitachi). The results are shown in Table 2. L-Methionine, which was not detected for the W3110 strain, was increased in the WABC strain and the WABCΔJ strain. Concerning the *metK* mutant strains, while the amount of L-methionine decreased in the WABCΔJK-2 strain,
20 a comparable amount was observed in the WABCΔJK-32 strain, and the amount was increased in the WABCΔJK-24 strain. Thus, the effect on the L-methionine production was observed. The WABCΔJK-24 strain harboring the plasmid pMWPthrmetA-W was given a private number AJ13425,
25 and it was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade

and Industry (postal code 305-8566, 1-3 Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, Japan) on May 14, 1998 as an accession number of FERM P-16808, and transferred from the original deposit to international deposit based on
 5 Budapest Treaty on September 27, 1999, and has been deposited as deposition number of FERM BP-6895.

Table 2: L-methionine production amount of wild-type *metA* introduced strains

Strain	Production amount (g/l)
W3110/pMWPthrmetA-W (<i>metA</i> [°])	0.000
WΔBC/pMWPthrmetA-W (<i>thrBC</i> ⁻ , <i>metA</i> [°])	0.008
WΔBCΔJ/pMWPthrmetA-W (<i>metBL</i> [°] , <i>thrBC</i> ⁻ , <i>metA</i> [°])	0.022
WΔBCΔJK-2/pMWPthrmetA-W (<i>thrBC</i> ⁻ , <i>metJ</i> ⁻ , <i>metBL</i> [°] , <i>metK</i> ¹ , <i>metA</i> [°])	0.014
WΔBCΔJK-24/pMWPthrmetA-W (<i>thrBC</i> ⁻ , <i>metJ</i> ⁻ , <i>metBL</i> [°] , <i>metK</i> ¹ , <i>metA</i> [°])	0.141
WΔBCΔJK-32/pMWPthrmetA-W (<i>thrBC</i> ⁻ , <i>metJ</i> ⁻ , <i>metBL</i> [°] , <i>metK</i> ¹ , <i>metA</i> [°])	0.023

10 *metK*¹: reduced *metK*, *metA*[°]: enhanced *metA*,
metBL[°]: enhanced *metBL*

Example 4: Acquisition of *metA* mutant strain and inhibition-desensitized type *metA* gene

15

The W3110 strain was inoculated to 2 ml of LB medium, and cultured at 37°C for 8 hours. 1 ml of the medium was centrifuged at 5,000 rpm for 10 minutes, and the cells were washed twice with 0.9% saline. The
 20 obtained cells were suspended in 100 μl of 0.9% saline,

5 μ l of which was inoculated to 5 ml of Davis-Mingioli minimal medium containing 1 g/l of α -methyl-DL-methionine (MM), and cultured at 37°C for 3 days. The medium was properly diluted, plated on Davis-Mingioli minimal medium containing 1 g/l of MM, and cultured at 37°C overnight. Some of grown colonies were subjected to colony separation on LB agar medium, and their growth was confirmed again on Davis-Mingioli minimal medium containing 1 g/l of MM. This procedure was independently performed 9 times, and six independent resistant strains, each designated as WMM4, WMM5, WMM6, WMM7, WMM8, and WMM9, were obtained.

Chromosomal DNA was prepared from these resistant strains. PCR was performed by using each chromosomal DNA as a template and primers having the sequences represented as SEQ ID NOS: 21 and 22 to amplify the *metaA* gene. The nucleotide sequence of each amplification fragment was determined by using primers for amplification of which nucleotide sequences are represented as SEQ ID NOS: 21 and 22, and primers having the sequences represented as SEQ ID NOS: 23 and 24. As for the *metaA* nucleotide sequence of the resistance strains, in the nucleotide sequence of wild-type *metaA* represented as SEQ ID NO: 25, thymine at the position of 887 was changed to guanine in the WMM4 strain, cytosine at the position of 893 was changed to thymine in the WMM5 strain, was the wild-type sequence was found in the

WMM6 strain, a sequence of ATCTC corresponding the 886th to the 890th nucleotides iterated twice, and an insertion sequence consisting of about 1300 nucleotides, called IS2 (Ghosal, D. et al., *Nucleic Acids Res.*, 6, 1111-1122 (1979)), was present between the repeated sequences in the WMM7 and WMM8 strains, and cytosine at the position of 79 was changed to thymine in the WMM9 strain. As a result, it was found that, in the amino acid sequence of HTS represented as SEQ ID NO: 26, the 296th isoleucine was changed to serine in the WMM4 strain, proline at the position of 298 was changed to leucine in the WMM5 strain, proline at the position of 298 and subsequent amino acid residues were changed to a sequence of arginine-leucine-alanine-proline due to an insertion sequence in the WMM7 and WMM8 strains, and arginine at the position of 27 was changed to cysteine in the WMM9 strain.

The strains WMM4, WMM5, WMM9 and WMM7, in which a mutation was observed in the *metA* structural gene, were each cultured in LB medium contained in a test tube at 37° overnight, and 1 ml of the medium was centrifuged at 5,000 rpm for 10 minutes. The cells were washed twice with 1 ml of 0.9% saline, and suspended in 1 ml of 0.9% saline, 0.5 ml of which was inoculated to 50 ml of minimal medium and cultured at 37°C for one day. The medium was centrifuged at 8,000 rpm for 10 minutes, and the cells were washed twice with 1 ml of 0.9% saline.

The obtained cells were suspended in 3 ml of 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM dithiothreitol, and a crude enzyme extract was obtained in the same manner as in Example 1. The HTS activity in the crude enzyme extract was measured with the reaction composition mentioned in Example 1 in the presence of an inhibitor. The results are shown in Table 3. The activity was undetectable for the WMM7 strain, and this was considered to reflect the marked decrease of the specific activity due to the amino acid sequence change caused by the insertion sequence. The specific activities of the other strains were about 1/4 of the wild strain. The inhibition by MM was canceled in all of the WMM4, WMM5, and WMM9 strains, and the inhibition by L-methionine was also reduced considerably. While the inhibition by SAM was hardly canceled in the WMM9 strain, tendency of cancellation was observed in the WMM4 and WMM5 strains. Inhibition by the combination of L-methionine and SAM, which exhibited the strongest inhibition for the wild-type HTS activity, was also markedly reduced in the WMM4 and WMM5 strains

Table 3: Activity of HTS derived from MM resistant strains in the presence of various inhibitors

Inhibitor	HTS activity (mmol/min/mg protein)				
	W3110	WMM9	WMM4	WMM5	WMM7
No addition	22.3	5.0	4.5	4.5	0.0
0.1 mM MM	18.6	4.9	4.1	4.6	0.0
1 mM MM	7.0	2.7	4.6	4.8	0.0
0.1 mM Met	14.3	2.5	4.5	4.2	0.0
1 mM Met	0.8	2.2	4.0	4.0	0.0
0.1 mM SAM	17.0	1.1	4.6	3.6	0.0
1 mM SAM	3.0	0.5	2.6	3.3	0.0
0.1 mM SAM + 0.1 mM Met	0.0	0.9	5.6	2.8	0.0

Example 5: L-Methionine production by

5

introduction of mutant *metaA*

PCR was performed by using chromosomal DNA from each of the WMM9, WMM4 and WMM5 strains among the *metaA* mutants obtained in Example 4 as a template, and

10 oligonucleotides having the sequences of SEQ ID NO: 21 and SEQ ID NO: 22 as primers to amplify a fragment containing the *metaA* gene. This amplification fragment had recognition sequences for *SphI* and *SalI* at the both ends. The both ends of this amplified fragment were

15 digested with *SphI* and *SalI*, and cloned into pHSG398 digested with *SphI* and *SalI*. The nucleotide sequence of each insert fragment was determined to confirm the mutation point. A *SphI* and *SalI* digestion product of this plasmid, *HindIII* and *SphI* digestion product of the

20 threonine promoter mentioned in Example 1, and pMW118 (Nippon Gene) digested with *HindIII* and *SalI* were mixed

and ligated. The JM109 strain was transformed with this ligation solution, and plasmids were extracted from the transformants. From the obtained plasmids, those comprising ligated three components were selected, and
5 designated as pMWPthrmetaA-9, pMWPthrmetaA-4, and pMWPthrmetaA-5, respectively.

Further, in order to obtain combination of the mutation points of each mutant *metaA* gene, site-specific mutagenesis was performed by using Mutan-Super Express
10 Km (TAKARA SHUZO) according to the instruction of the manufacturer. pMWPthrmetaA-9+4 were produced by combining the *metaA*-4 mutation with the *metaA*-9 mutation using an oligonucleotide having the sequence of SEQ ID NO: 27. pMWPthrmetaA-9+5 was similarly produced by
15 combining the *metaA*-5 mutation with the *metaA*-9 mutation. Furthermore, pMWPthrmetaA-9+4+5 was produced by combining the *metaA*-9 mutation with the *metaA*-4 and *metaA*-5 mutations using an oligonucleotide having the sequence of SEQ ID NO: 28.

20 The W Δ BC Δ JK-32 strain was transformed with these plasmids to obtain transformants. Each of the transformants was cultured at 37°C overnight on an LB plate containing 50 mg/l of ampicillin. The cells were inoculated to 20 ml of medium at pH 7 containing 40 g/l
25 of glucose, 1 g/l of magnesium sulfate, 16 g/l of ammonium sulfate, 1 g/l of potassium dihydrogenphosphate, 2 g/l of yeast extract (Bacto Yeast-Extract, Difco),

0.01 g/l of manganese sulfate, 0.01 g/l of iron sulfate, 30 g/l of calcium carbonate, 50 mg/l of ampicillin, and 0.5 g/l of L-threonine, and cultured at 37°C for 48 hours. The cells were separated from the culture, and the amount of L-methionine was measured by an amino acid analyzer (Hitachi). The results are shown in Table 4. The amount of L-methionine accumulation increased several times in the strains introduced with a mutant *metA*, compared with the strain introduced with a wild-type *metA*. Furthermore, by combining mutations, further increase of L-methionine production amount was obtained.

Table 4: L-methionine production amount of mutant *metA*-introduced strains

Strain	Amount of L-methionine production (g/l)
W Δ BC Δ Δ JK-32/pMWPthr <i>metA</i> -W	0.023
W Δ BC Δ JK-32/pMWPthr <i>metA</i> -9	0.158
W Δ BC Δ JK-32/pMWPthr <i>metA</i> -4	0.108
W Δ BC Δ JK-32/pMWPthr <i>metA</i> -5	0.131
W Δ BC Δ JK-32/pMWPthr <i>metA</i> -9+4	0.206
W Δ BC Δ JK-32/pMWPthr <i>metA</i> -5+9	0.207
W Δ BC Δ JK-32/pMWPthr <i>metA</i> -9+4+5	0.236